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DECLARATION OF DOROTHY FRENCH UNDER 37 C.F.R. § 1.132

I, Dorothy French, hereby declare that:

1. I am an inventor of the subject matter described and claimed in the above-identified United States patent application 10/516,868, along with Avi J. Ashkenazi, Jean-Philippe F. Stephan, and Luc Desnoyer.

2. I am a co-author of a paper published in the American Journal of Pathology, Vol. 160, No.6 June 2002 and entitled "A mouse model of hepatocellular carcinoma: ectopic expression of fibroblast growth factor 19 in skeletal muscle of transgenic mice" ("the Nicholes paper"), a copy of which is provided herewith as Appendix A. The other co-authors are Katrina Nicholes, Susan Guillet, Elizabeth Tomlinson, Kenneth Hillan, Barbara Wright, Gretchen D. Frantz, Thunh A. Pham, Lisa Dillard-Telm, Siao Ping Tsai, Jean-Philippe Stephan, Jeremy Stinson, and Timothy Stewart.

3. Jean-Philippe Stephan and I are the only authors who inventively contributed to the subject matter disclosed in the Nicholes paper. The other authors, Katrina Nicholes, Susan Guillet, Elizabeth Tomlinson, Kenneth Hillan, Barbara Wright, Gretchen D. Frantz, Thunh A. Pham, Lisa Dillard-Telm, Siao Ping Tsai, Jeremy Stinson, and Timothy Stewart, performed various tasks under our direction and control (e.g., provision of the FGF19 transgenic mouse). They made no inventive contribution to the subject matter of the Nicholes paper or to the claims of this application.

4. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information or belief are believed to be true, and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both under Section 1001 of Title 18 of the United States Code and that such willful statements may jeopardize the validity of the application or any patent issued thereon.

Date: _____

By: _____

Dorothy French

Animal Model

A Mouse Model of Hepatocellular Carcinoma

Ectopic Expression of Fibroblast Growth Factor 19 in Skeletal Muscle of Transgenic Mice

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Most mouse models of hepatocellular carcinoma have expressed growth factors and oncogenes under the control of a liver-specific promoter. In contrast, we describe here the formation of liver tumors in transgenic mice overexpressing human fibroblast growth factor 19 (FGF19) in skeletal muscle. FGF19 transgenic mice had elevated hepatic α -fetoprotein mRNA as early as 2 months of age, and hepatocellular carcinomas were evident by 10 months of age. Increased proliferation of pericentral hepatocytes was demonstrated by 5-bromo-2'-deoxyuridine incorporation in the FGF19 transgenic mice before tumor formation and in nontransgenic mice injected with recombinant FGF19 protein. Areas of small cell dysplasia were initially evident pericentrally, and dysplastic/neoplastic foci throughout the hepatic lobule were glutamine synthetase-positive, suggestive of a pericentral origin. Consistent with chronic activation of the Wingless/Wnt pathway, 44% of the hepatocellular tumors from FGF19 transgenic mice had nuclear staining for β -catenin. Sequencing of the tumor DNA encoding β -catenin revealed point mutations that resulted in amino acid substitutions. These findings suggest a previously unknown role for FGF19 in hepatocellular carcinomas. (*Am J Pathol* 2002, 160:2295–2307)

Most of the fibroblast growth factor (FGF) family members regulate cell proliferation, migration, and differentiation during development and in response to injury.¹ Notably,

several of the FGFs and their receptors are implicated in the development and progression of tumors. For example, FGF8 up-regulation is correlated with growth and invasiveness of human breast carcinomas^{2–4} and FGF8 isoforms are up-regulated in human prostate cancer.^{5–7} In addition, elevated circulating levels of FGF2 were demonstrated in patients with B-cell chronic lymphocytic leukemia and chronic myeloid leukemia.⁸ Mouse models overexpressing FGFs further implicate FGF family members in the pathogenesis of cancer. Transgenic mice overexpressing FGF8 under control of the mouse mammary tumor virus promoter develop breast tumors,⁹ and transgenic mice overexpressing FGF10 under the control of a lung-specific promoter form pulmonary adenomas.¹⁰ Mutations or overexpression of FGF receptors have also been implicated in neoplastic transformation. Up-regulation of FGFR4 is described in mammary fibroadenomas,¹¹ FGFR3 is constitutively activated in multiple myeloma,¹² and activating mutations were identified in FGFR2 and FGFR3 from gastric and colorectal cancers.¹³

The role of FGF19 in cancer is unknown. FGF19 is a novel member of the FGF family with unique specificity for FGFR4.¹⁴ Unlike other FGF family members, FGF19 has minimal mitogenic activity on fibroblasts *in vitro*. To understand the *in vivo* effects of FGF19, we generated transgenic mice overexpressing FGF19 in skeletal muscle. We describe here a unique model of hepatocellular carcinoma (HCC) that developed in FGF19 transgenic mice by 10 months of age.

Primary cancer of the liver, or HCC, is the third most frequent cause of death by cancer in the world.¹⁵ Consistent with the observation that oncogenes, growth factors, or viral genes are frequently up-regulated in human HCC, it is not surprising that overexpression of these genes in the liver also causes HCC in transgenic mice. Transgenic mouse models of HCC previously described include overexpression of transforming growth factor- α

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Table 1. Gross and Histologic Changes in the Liver from FGF19 Transgenics

Age (months)	Sex	Gross evidence of tumor	Dysplasia (basophilic foci)	Carcinoma	Age-matched wild-type mice*
2 to 4	F	0/17	0/17	0/17	10
	M	0/7	0/7	0/7	7
7 to 9	F	0/15	5/15 (33%)	0/15	14
	M	0/15	1/15 (7%)	0/15	14
10 to 12	F	8/10 (80%)	7/10 (70%)	8/10 (80%)	11
	M	2/9 (22%)	7/9 (77%)	2/9 (22%)	7

Incidence is expressed as number of animals with indicated parameter over the total number of animals in the group.

*Total numbers of age-matched wild-type mice are indicated in the last column although no dysplastic or neoplastic changes were found in this group.

(TGF- α) alone¹⁶⁻¹⁹ or in combination with *c-myc*,²⁰ mutated H-ras,²¹ hepatitis B viral genes encoding HbsAg and HBx,^{22,23} and SV40 large T antigen.^{24,25} Liver-specific promoters drive transgene expression in all of these models. To our knowledge, no mouse model has been described in which ectopic expression of an oncogene or growth factor leads to hepatic tumors.

In the normal liver, hepatocytes are mitotically quiescent but can readily proliferate in response to injury.²⁶ Several studies investigating the pathogenesis of HCCs reveal that constitutive hepatocellular proliferation is a prerequisite for transformation.²⁷ Hepatocellular proliferation leading to transformation may be initiated by inflammation. For example, transgenic mice overexpressing the hepatitis B virus large envelope protein develop focal hepatocellular necrosis because of excessive accumulation of protein within the endoplasmic reticulum, followed by inflammation that precedes tumor formation.^{28,29} Inflammation is also prominent in mice lacking the *mdr2* gene that results in failure to transport phosphatidyl choline into the bile and inability to emulsify biliary components leading to inflammation, hepatocellular proliferation, and HCC by 18 months of age.³⁰ Similarly, mice lacking peroxisomal fatty acyl-CoA oxidase develop hepatitis followed by hepatocellular regeneration then hepatocellular tumors by 15 months of age.³¹ Alternatively, in the absence of inflammation, increased hepatocellular proliferation and subsequent transformation can result from genomic alteration. For example, insertional activation of an oncogene leading to hepatocellular proliferation before HCC was demonstrated in woodchuck hepatitis virus infection. HCC in woodchucks results from integration of woodchuck hepatitis virus at the *c-myc* or *n-myc2* locus.³²⁻³⁴ Tumor induction without preceding inflammation or necrosis also occurs in transgenic mice overexpressing TGF- α ,^{19,20,35,36} *c-myc*,²⁰ *c-Ha-ras*,²¹ or SV40 large T antigen.^{37,38}

The results of this study indicate that tumors arise from pericentral hepatocytes after increased proliferation and dysplasia. In addition, increased proliferation is accompanied by expression of α -fetoprotein (AFP), an oncofetal protein used as a marker for neoplastic transformation of hepatocytes,³⁹ before occurrence of tumors. Similar to mice overexpressing TGF- α and/or *c-myc*, early dysplastic foci are predominantly small-cell type in the FGF19 transgenic mice. In contrast, neither *c-myc* nor *tgf- α* mRNA was elevated in liver tumors from the FGF19 transgenic mice. Nuclear accumulation of β -catenin was evi-

dent in neoplastic cells from some of the FGF19 liver tumors suggesting nuclear translocation of β -catenin and activation of the Wingless/Wnt signaling pathway. This is the first report implicating an FGF family member in development of hepatocellular tumors and this model may provide insight into the pathogenesis of human HCC.

Materials and Methods

Generation of FGF19 Transgenic Mice

All animal protocols were approved by an Institutional Animal Care and Use Committee. Construction of the FGF19 transgenic mice was previously described.⁴⁰ Briefly, the human FGF19 cDNA¹⁴ was ligated 3' to the pRK splice donor/acceptor site that was preceded by the myosin light chain.⁴¹ The FGF19 cDNA was also followed by the splice donor/acceptor sites present between the fourth and fifth exons of the human growth hormone gene.⁴² The entire expression fragment was purified free from contaminating vector sequences and injected into one-cell mouse eggs derived from FVB \times FVB matings. Transgenic mice were identified by polymerase chain reaction (PCR) analysis of DNA extracted from tail biopsies using DNeasy and PCR core kits (Qiagen, Valencia, CA). Expression of FGF19 was determined by real-time reverse transcriptase-PCR (TaqMan; Perkin Elmer, Emeryville, CA) on total RNA from skeletal muscle biopsies.

Gross and Histopathological Analyses

To determine the onset of liver changes in FGF19 transgenic mice, transgenic and wild-type mice were evaluated at designated intervals throughout the course of a year (Table 1). Five to eight each of male and female FGF19 transgenic mice and wild-type littermates were euthanized every month and evaluated as indicated below. Body and liver weights were recorded. Livers were examined for gross lesions. Specimens from each lobe and from grossly visible tumors were fixed in 10% buffered formalin, embedded in paraffin, sectioned at 4 μ m, and used for immunohistochemistry, *in situ* hybridization, or stained with hematoxylin and eosin (H&E). For *in vivo* labeling of S-phase hepatocytes, 5-bromo-2'-deoxyuridine (BrdU; Sigma Chemical Co., St. Louis, MO) was dissolved in phosphate-buffered saline (PBS) by heating

at a concentration of 100 mg/ml. While still warm, BrdU solution was injected into osmotic minipumps (ALZET model 1002) and incubated in excess PBS for 4 hours in amber vials. Pumps were subcutaneously implanted between the shoulder blades and left implanted for 6 days. For molecular analysis, half of each tumor was quickly frozen in liquid nitrogen and stored at -80°C .

Measurement of Serum FGF19 Protein

Human FGF19 was measured in serum from transgenic mice using an enzyme-linked immunosorbent assay. The 96-well Nunc-Immuplates (Nalge Nunc Intl. Corp., Rochester, NY) were coated at 4°C overnight with a mouse monoclonal antibody anti-rhFGF19 (mAb 1A6; Genentech, Inc., South San Francisco, CA) at $2\text{ }\mu\text{g/ml}$ in carbonate buffer (pH 9.6). Enzyme-linked immunosorbent assay plates were washed with PBS and 0.05% Tween-20 (pH 7.2) and blocked for 2 hours with PBS, 0.05% Tween-20, and 0.5% bovine serum albumin (pH 7.2). Serum samples and rhFGF19 standard were diluted in PBS, 0.5% bovine serum albumin, 0.2% bovine IgG, 0.25% CHAPS, 5 mmol/L ethylenediaminetetraacetic acid (pH 7.4), 0.05% Tween-20, and 0.35 mol/L NaCl and incubated on the enzyme-linked immunosorbent assay plate for 2 hours. After washing with PBS and 0.05% Tween-20 (pH 7.2), the enzyme-linked immunosorbent assay plates were incubated with a secondary biotinylated monoclonal anti-rhFGF19 (mAb 2A3, Genentech, Inc.) antibody for 1 hour before washing, followed by incubation with Amersham streptavidin-horseradish peroxidase (Amersham Pharmacia Biotech, Piscataway, NJ). Signal was revealed using the chromogenic substrate TMB (Kirkegaard & Perry Laboratories, Gaithersburg, MD) and read at 450/620 nm after addition of phosphoric acid (1 mol/L).

In Situ Hybridization

^{33}P -labeled murine FGFR4 and AFP riboprobes were used to evaluate gene expression in murine liver, lung, spleen, kidney, and brain. To generate the probes, PCR primers were designed to amplify either a 654-bp fragment of murine AFP spanning from nucleotides 731 to 1385 of NM.007423 (upper: 5' CCTCCAGGCA ACAACCATTA T; lower, 5' CCGGTGAGGT CGATCAG) or a 170-bp fragment of murine FGFR4 spanning from nucleotides 327 to 497 of NM.008011 (upper: 5' CGAGTACGGGTTGGAGA; lower: 5' TGCTGAGTGTCTTGGGGTCTT). Primers included extensions encoding 27-nucleotide T7 or T3 RNA polymerase initiation sites to allow *in vitro* transcription of sense or anti-sense probes, respectively, from the amplified products.⁴³ Sections were deparaffinized, deproteinized in $4\text{ }\mu\text{g/ml}$ of proteinase K for 30 minutes at 37°C , and further processed for *in situ* hybridization as previously described.⁴⁴ ^{33}P -UTP labeled sense and anti-sense probes were hybridized to the sections at 55°C overnight. Unhybridized probe was removed by incubation in $20\text{ }\mu\text{g/ml}$ of RNase A for 30 minutes at 37°C ,

followed by a high stringency wash at 55°C in $0.1\times$ standard saline citrate for 2 hours, and dehydration through graded ethanols. The slides were dipped in NBT2 nuclear track emulsion (Eastman Kodak, Rochester, NY), exposed in sealed plastic slide boxes containing desiccant for 4 weeks at 4°C , developed, and counterstained with H&E.

Immunohistochemical and Morphometric Analyses

Monoclonal antibodies to glutamine synthetase (Chemicon, Temecula, CA) and β -catenin (Transduction Laboratories, Lexington, KY) were prelabeled using the mouse-on-mouse Iso IHC Kit (InnoGenex, San Ramon, CA) following the manufacturer's instructions. Pretreatment of all slides included antigen retrieval in preheated DAKO Target Retrieval (DAKO, Carpinteria, CA) for 20 minutes at 99°C and endogenous peroxidase blocking by KPL Blocking Solution (Kirkegaard & Perry) for 4 minutes at room temperature. After PBS washing, the endogenous biotin was blocked using an Avidin Blocking Kit (Vector Laboratories, Burlingame, CA) and the endogenous proteins were blocked using Power Block Reagent (InnoGenex, San Ramon, CA). The sections were incubated with the prelabeled primary antibodies for 60 minutes at room temperature and washed in PBS. Sections were then labeled with Vectastain Elite avidin biotin complex (ABC)-peroxidase (Vector Laboratories) followed by tyramide amplification (NEN Life Science Products, Boston, MA) and visualization using metal enhanced diaminobenzidine (Pierce, Rockford, IL). Murine IgG (Oncogene, Cambridge, MA) was used as an isotype control; normal liver and cell pellets were used to determine tissue and antigen specificity.

Cellular proliferation was evaluated using a monoclonal antibody to BrdU (clone IU-4; Caltag Laboratories, Burlingame, CA). After deparaffinization, sections were treated with preheated 2 N HCl for 30 minutes at 37°C , rinsed with borate buffer (pH 7.6) for 1 minute, and digested in preheated 0.01% trypsin (Sigma) for 3 minutes at 37°C . Endogenous peroxidase and endogenous biotin were blocked as previously described. Endogenous proteins were blocked with 10% normal horse serum (Life Technologies, Inc., Rockville, MD) in 3% bovine serum albumin and PBS (Boehringer Mannheim, Indianapolis, IN) for 30 minutes. The sections were then incubated with anti-BrdU antibody for 60 minutes, followed by biotinylated horse anti-mouse IgG, Vectastain Elite ABC-Peroxidase (Vector Laboratories), then Immunopure metal-enhanced diaminobenzidine substrate (Pierce) for visualization. For morphometric analysis of BrdU-labeled sections, 1000 to 3000 hepatocytes were counted for each animal using MetaMorph image analysis software (Universal Imaging Corp., Downingtown, PA). The labeling index denotes the number of BrdU-positive hepatocytes divided by the total number of hepatocytes counted and indicated as a percentage.

Table 2. Primers and Probes Used for Real-Time Reverse Transcriptase-PCR Analysis of Growth Factors and Oncogenes

Gene name	Forward primer 5'-3'	Reverse primer 5'-3'	Probe 5'-3'
Murine RPL19	GCG CAT CCT CAT GGA GCA CA	GGT CAG CA GGA GCT TCT TG	CAC AAG CTG AAG GCA GAC AAG GCC C
Murine TGF- α	CAG CTT TAG TGT CCG AAC GGT	GTG TAC AGG CAC TGG GAT GGT	CCA GCC AGT CGC AGC AGC CA
Murine <i>c-myc</i>	AGC AAC AAC CGC AAG TGC T	GTC CGC CTC TTG TCG TTT TC	CAG CCC CAG GTC CTC AGA CAC GG
Murine AFP	GAA GTG GAT CAC ACC CGC TT	TTT TCG TGC AAT GCT TTG GA	CCT CAT CCT CCT GCT ACA TTT CGC TGC
Murine hepatocyte growth factor	CCT GAC ACC CCT TGG GAG T	GTT TCC ATA GGG ACA TCA GTC TCA T	TTG TGC AAT TAA AAC GTG CGC TCA CAG T
Murine FGFR4	CGG GAC TAG CTG CAA AAC T	TGA AGT CCC AAG GCC TCT A	TGC TCT AAA CAT TTC TAG TTC CCC CAA ACA

Gene Expression

Total RNA was extracted from frozen liver samples using RNA STAT-60 (Tel-test "B" Inc., Friendswood, TX). Liver samples were homogenized in the RNA STAT-60, incubated at room temperature for 5 minutes, and centrifuged at $12,000 \times g$ for 10 minutes at 4°C. Chloroform was added to the supernatant to extract the RNA, followed by isopropanol precipitation for 10 minutes. The pellet was washed with 75% EtOH and resuspended in diethyl pyrocarbonate-treated water. Total RNA was DNase-treated followed by addition of DNase inactivation reagent (Ambion) and centrifugation. Supernatant was used as RNA template for real-time PCR. RNA concentration was determined using a spectrophotometer (DU 530; Beckman) and visualized on a 1.2% agarose gel.

Primers and probes were designed using Primer Express 1.1 (PE Applied Biosystems) for murine RPL19, FGFR4, TGF- α , hepatocyte growth factor, *c-myc*, and AFP (Table 2). Amplification reactions (50 μ l) contained 100 ng of RNA template, 5 mmol/L of MgCl₂, 1 \times buffer A, 1.2 mmol/L of dNTPs, 2.5 U of TaqGold polymerase, 20 U of RNase inhibitor, 12.5 U of MuLV reverse transcriptase, 2 μ mol/L each forward and reverse primer, and 5 μ mol/L of probe (Perkin Elmer). Thermal cycle (Perkin Elmer ABI Prism 7700 sequence detector) conditions were 48°C for 30 minutes, 95°C for 10 minutes, and 95°C for 15 seconds, and 60°C for 1 minute for 40 cycles. Analyses of data were performed using Sequence Detector 1.6.3 (PE Applied Biosystems) and results for genes of interest were normalized to RPL19.

Recombinant FGF19 Protein

Recombinant human FGF19 (rFGF19) was expressed intracellularly in *Escherichia coli* as previously described.⁴⁰ The FGF19 protein was purified via anion exchange chromatography, size exclusion chromatography, and preparative reverse-phase chromatography. Sequence analysis and analysis by mass spectrometry indicated that purified rFGF19 had the expected mass and N-terminal sequence. Nontransgenic female FVB mice were injected intraperitoneally with 30 μ g of rFGF19 protein or ArgPO₄ vehicle in a volume of 100 μ l once daily for 6 days. BrdU was administered by Alzet minipumps placed subcutaneously on day 1, as described above, and the mice were necropsied on the day 6.

Cloning and Sequencing of β -Catenin

DNA extracted from tumor tissue was PCR-amplified using forward and reverse primers 5' TAC AGG TAG CAT TTT CAG TTC AC 3' and 5' TAG CTT CCA AAC ACA AAT GC 3', respectively. PCR products were subcloned into pCR2.1 using the TA cloning kit (Invitrogen, Carlsbad, CA). Sequencing of subcloned PCR products was done as outlined in the ABI Prism Big Dye Terminator Cycle Sequencing Ready Reaction Kit on an Applied Biosystems Prism 3700 DNA Analyzer. M13 primers were used for the TA vector. The trace files were edited and aligned using Sequencer (Gene Codes Corp., Ann Arbor, MI). Mutations were identified by comparing the trace files to the murine β -catenin sequence published in GenBank (NM007614).

Statistical Analysis

Data are presented as the means plus or minus standard deviations. Comparisons between transgenic and wild-type mice or protein-injected and vehicle control mice were made using an unpaired Student's *t*-test.

Results

Hepatocellular Dysplasia and Neoplasia in FGF19 Transgenic Mice

As early as 2 to 4 months of age hepatocytes adjacent to central veins formed a single columnar row with nuclei polarized away from the endothelial basement membrane of the central vein (Figure 1A) that was not observed in wild-type mice (Figure 1B). Dysplastic changes (areas of altered hepatocellular foci) preceded tumor formation and were evident by 7 to 9 months (Table 1). Within this age group, 33% of female and 7% of male transgenics had hepatocellular dysplasia without evidence of neoplasia. Interestingly, dysplastic foci were predominantly of the small-cell type and oriented around central veins (Figure 1C). Rarely, foci of large dysplastic hepatocytes were noted (Figure 1D). FGF19 transgenic mice developed liver tumors by 10 to 12 months of age at an overall frequency of 53% (Table 1). Within the 10- to 12-month-old group, 80% (8 of 10) of female and 22% (2 of 9) of male FGF19 transgenic mice had locally invasive HCCs (Figure 2). Tumors were solitary or multifocal, involving different liver lobes. Mean

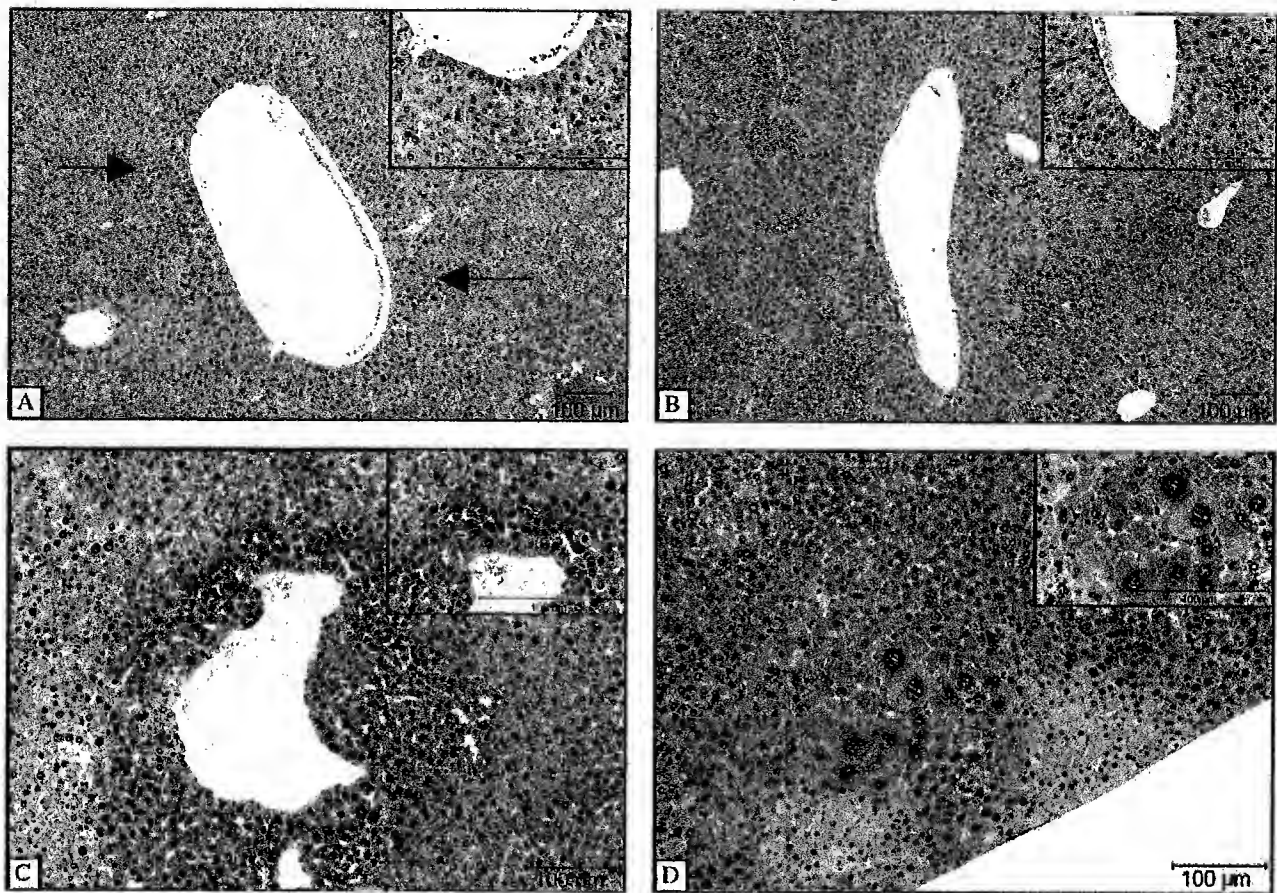


Figure 1. Preneoplastic hepatocellular changes in FGF19 transgenic mice. As early as 14 weeks of age pericentral hepatocytes formed a dense cluster around the central veins (arrows) with polarization of nuclei of the innermost hepatocytes away from the vessel lumen in FGF19 transgenics (A) that was not present in liver from nontransgenic littermate mice (B). Pericentral small dysplastic hepatocytes (C) were the predominant type of hepatocellular dysplasia although foci of large dysplastic hepatocytes (D) were occasionally noted. Arrows delineate areas of altered hepatocellular foci (shown at higher magnification in the insets). Original magnifications: $\times 100$ (A and B); $\times 400$ (C and D). Inset original magnifications: $\times 400$ (A and B); $\times 600$ (C and D).

liver weights in the 10- to 12-month-old female FGF19 transgenics were increased 30% relative to liver weights of wild-type mice (mean liver weight, 1.97 g and 1.54 g, respectively; $P < 0.01$) attributable to tumor mass. The mean liver weight for 10- to 12-month-old male FGF19 transgenic mice was not significantly different from wild-type mice, likely because of the low incidence of tumors in the male transgenic mice (mean liver weight, 1.53 g and 1.63 g, respectively; $P = 0.37$). Histologically, neoplastic hepatocytes invaded and replaced adjacent normal hepatic parenchyma (Figure 2B). HCCs in the FGF19 transgenic mice were predominantly the solid type although a trabecular pattern was occasionally noted. Figure 2C shows the typical morphology of neoplastic hepatocytes: neoplastic cells with nuclear pleomorphism and frequent mitoses (Figure 2C, arrows). The tumors did not metastasize. Other tissues evaluated histologically included: lungs, heart, spleen, kidneys, bone (femur), intestines, brain, pituitary gland, thyroid glands, and skeletal muscle. Despite the fact that FGF19 was expressed in the skeletal muscle, no histological changes were evident in that tissue.

Serum FGF19 protein levels were determined in the FGF19 transgenic and wild-type mice to assess whether

phenotypic differences between male and female transgenic mice could be because of differences in levels of protein expression. The 2- to 4-month-old transgenic females ($n = 16$) have mean serum FGF19 protein levels of 77.7 ng/ml and the transgenic males in the same age group ($n = 7$) have mean serum FGF19 protein levels of 63.2 ng/ml ($P = 0.07$). FGF19 serum protein levels are considerably lower in the older animals. The 7- to 9-month-old transgenic females ($n = 10$) have mean serum FGF19 protein levels of 21.8 ng/ml and transgenic males in the same age group ($n = 13$) have mean serum FGF19 protein levels of 18.6 ng/ml ($P = 0.20$). The 10- to 12-month-old transgenic females ($n = 9$) have mean FGF19 serum protein levels of 24.3 ng/ml and the transgenic males in the same age group have mean FGF19 serum protein levels of 20.7 ng/ml ($P = 0.41$).

FGFR4, the Receptor for FGF19, Is Expressed in Murine Liver.

FGF19 was previously shown to selectively bind with high affinity to FGFR4.¹⁴ Although FGFR4 expression has been

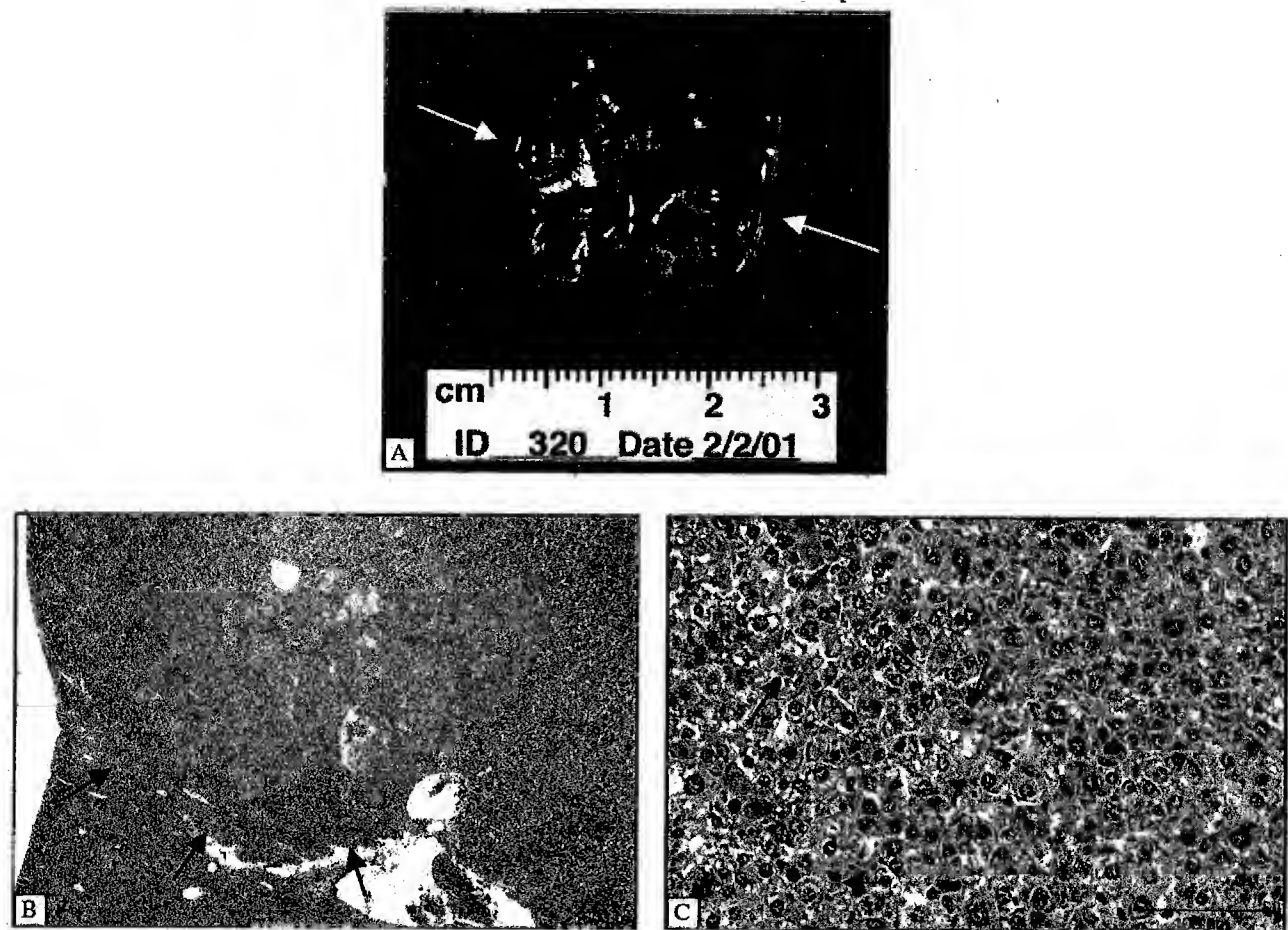


Figure 2. Hepatocellular neoplasia in FGF19 transgenic mice. **A:** Multiple, large, raised tumors protrude from the hepatic surface of the liver from a 10-month-old FGF19 transgenic mouse (arrows). **B:** Histologically, neoplastic cells invade and replace normal hepatic architecture and are arranged in solid sheets or cords. Arrows mark the border of the tumor and adjacent normal liver. **C:** Pleomorphism of neoplastic hepatocytes and atypical mitotic figures (arrows). Original magnifications: $\times 40$ (B); $\times 400$ (C).

demonstrated in mouse and rat hepatocytes,^{45–47} we used *in situ* hybridization with a ³³P-labeled murine FGFR4 riboprobe to determine expression patterns in wild-type and FGF19 transgenic mice. In both wild-type and FGF19 transgenic mice, a strong signal for murine *fgfr4* mRNA was present in hepatocytes adjacent to central veins and in random, small hepatocytes throughout the lobule (Figure 3). There was not a significant difference in signal intensity or distribution based on genotype. In addition, real-time reverse transcriptase-PCR did not demonstrate any difference in levels of *fgfr4* mRNA between FGF19 transgenic and wild-type mice (data not shown).

FGF19 Transgenic Mice and rFGF19 Protein-Injected Mice Showed Increased Hepatocellular Proliferation

Constitutive hepatocellular proliferation is considered a prerequisite for neoplastic transformation.²⁷ Therefore, we used *in vivo* BrdU labeling in the FGF19 transgenic mice to assess hepatocellular proliferation. Labeled hepatocytes were predominantly perivenular (Figure 4B) whereas BrdU-labeled hepatocytes were

rare in wild-type mice (Figure 4A). By 2 to 4 months of age the BrdU-labeling index of hepatocytes was eightfold higher in FGF19 transgenic females than age-matched wild-type females ($P = 0.00003$) and twofold to threefold higher in FGF19 transgenic males than age-matched wild-type males ($P = 0.040$) (Figure 4C). The labeling index is also increased twofold to threefold in 7- to 9-month-old female and male FGF19 transgenics relative to their respective controls ($P = 0.0000002$ and $P = 0.006$, respectively; Figure 4D). Together these data indicate hepatocellular proliferation precedes tumor development and that the proliferative fraction is predominantly pericentral hepatocytes.

To determine whether hepatocellular proliferation was because of acute effects of FGF19 *in vivo*, we injected the purified protein into nontransgenic female mice while infusing BrdU throughout a period of 6 days. Mice receiving rFGF19 protein had a significantly higher BrdU-labeling index than mice receiving vehicle alone ($P = 0.014$). Similar to results described above in FGF19 transgenic mice, rFGF19-injected mice have a threefold to fivefold increase in hepatocellular proliferation relative to vehicle-injected mice (Figure 4E).

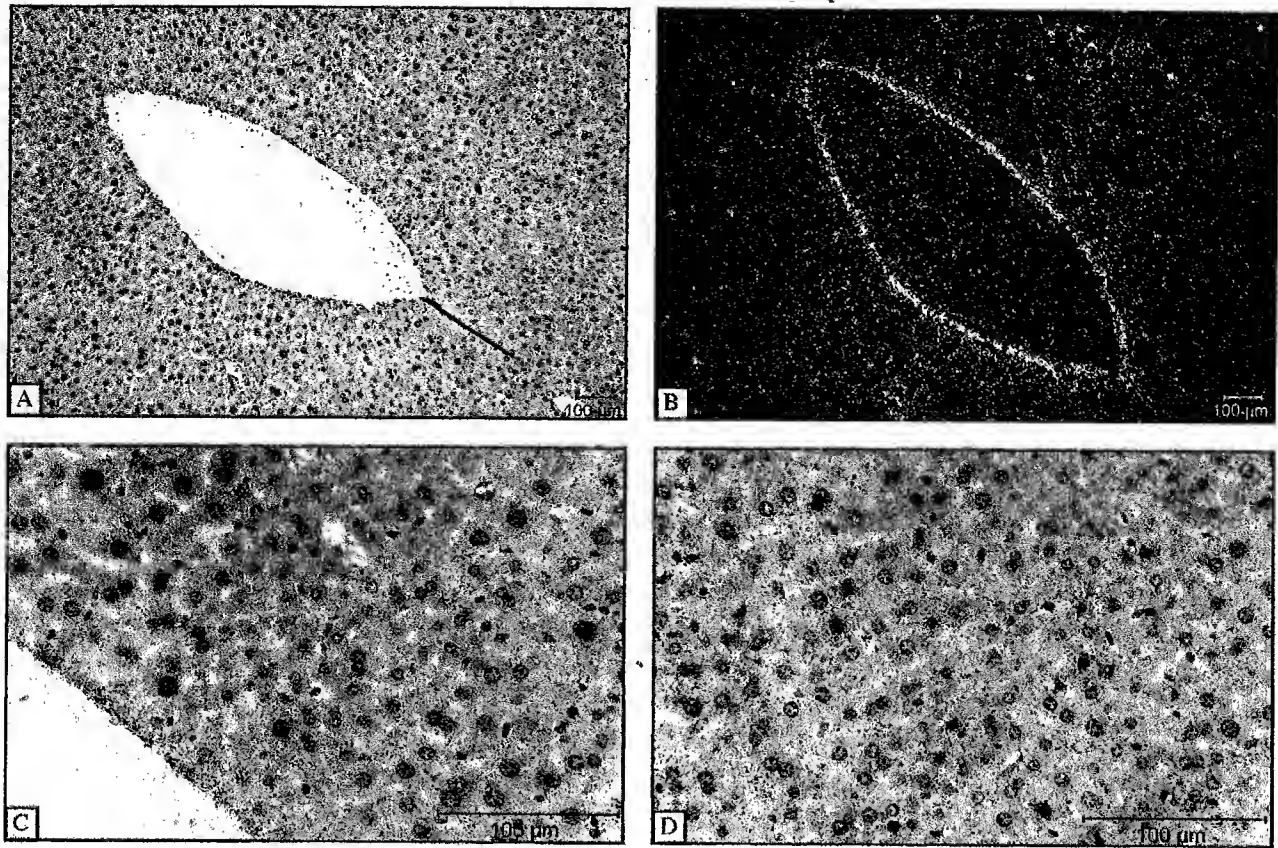


Figure 3. FGFR4 expression in murine liver. Bright-field (A) and dark-field (B) illumination of *in situ* hybridization with a murine FGFR4 riboprobe showing expression in perivenular and random hepatocytes. C and D: Higher magnification of bright-field demonstrating silver grains over random small hepatocytes. Original magnifications: $\times 100$ (A and B); $\times 400$ (C and D).

Pericentral Hepatocytes Give Rise to Neoplastic Foci

Glutamine synthetase is a marker for tracing hepatocellular lineage during preneoplastic and early neoplastic stages.⁴⁸ In the 10- to 12-month-old mice, 10 of 19 FGF19 transgenic mice had HCCs. All of the FGF19-induced tumors were strongly positive for glutamine synthetase by immunohistochemistry (Figure 5A). In contrast, liver from wild-type mice showed the expected pattern of staining one to three cell layers of pericentral hepatocytes (Figure 5, B and D). Foci of large dysplastic hepatocytes were also glutamine synthetase-positive (Figure 5C). Glutamine synthetase immunoreactivity of the neoplastic cells suggests they originated from the one to three cell layers of hepatocytes around the central veins that constitutively express glutamine synthetase.

AFP is an oncofetal protein expressed by neoplastic hepatocytes but not normal adult hepatocytes and is used as an indicator of neoplastic transformation in the liver.³⁹ Real-time reverse transcriptase-PCR showed hepatic AFP mRNA was elevated in FGF19 transgenic mice relative to wild types (Figure 6, A and B). At 2 to 4 months of age female transgenics had a 13-fold increase ($P = 0.01$) and male transgenics had an 18-fold increase ($P = 0.005$) in AFP expression relative

to respective wild-type controls. The 7- to 9-month-old transgenic females had a fourfold increase ($P = 0.01$) and males had a threefold increase ($P = 0.03$) in AFP expression relative to respective wild-type controls. Subsequently, we evaluated AFP expression by *in situ* hybridization to determine which cells were expressing AFP before tumor formation. Consistent with our previous findings that indicated initial involvement of pericentral hepatocytes, AFP expression was demonstrated in hepatocytes adjacent to central veins (Figure 6, C and D). Neoplastic hepatocytes also consistently expressed AFP (Figure 6, E and F).

Expression of Growth Factors and Oncogenes in FGF19 Transgenic Mice

To investigate potential mechanisms of hepatocarcinogenesis, we evaluated expression of mRNA encoding TGF- α , hepatocyte growth factor, and *c-myc*. Overexpression of TGF- α alone^{16,18} or in combination with *c-myc*²⁰ in the liver of transgenic mice leads to tumor development. In this study, real-time reverse transcriptase-PCR analysis at each time point did not demonstrate up-regulated expression of mRNA encoding TGF- α , hepatocyte growth factor, or *c-myc* in liver from MLC.FGF19 transgenic relative to age-matched wild-type mice (data not shown).

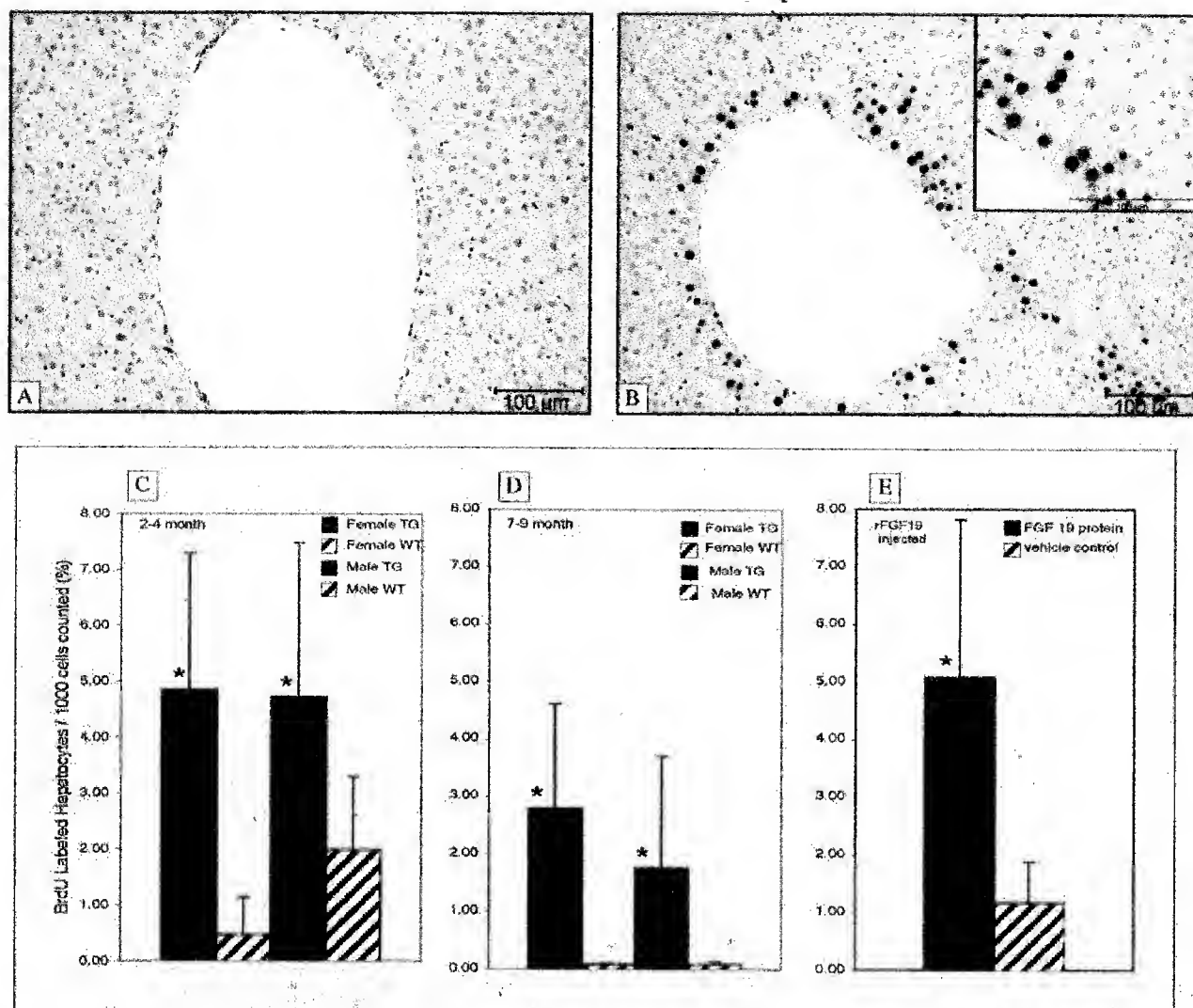


Figure 4. Increased proliferation of pericentral hepatocytes in FGF19 transgenics. Immunostaining for BrdU after a 5-day infusion by osmotic minipump of liver from a wild-type (A) and a FGF19 transgenic mouse (B). Morphometric analysis of BrdU-immunostained sections from FGF19 transgenics compared to wild-type mice: 2 to 4 months (C), 7 to 9 months (D), and FGF19-injected mice (E). The labeling index denotes the number of BrdU-positive hepatocytes divided by the total number of cells counted and indicated as a percentage. *, $P < 0.05$. Original magnifications: $\times 200$ (A and B), $\times 600$ (inset in B).

β -Catenin Activation and Somatic Mutations in MLC.FGF19 HCCs

To further evaluate the molecular pathogenesis of HCCs in FGF19 transgenic mice, we used immunohistochemical staining for β -catenin in addition to cloning and sequencing exon 2 of the β -catenin gene from tumor tissue. HCCs from nine different FGF19 transgenic mice were evaluated for immunoreactivity to β -catenin antibody. Four of the nine tumors (44%) had nuclear and cytoplasmic staining for β -catenin in neoplastic hepatocytes (Figure 7, A and B). All four tumors with β -catenin immunoreactivity were from female FGF19 transgenic mice in the 10- to 12-month age group. Cloning and sequencing hepatic DNA encoding exon 2 of β -catenin from tumor tissue that was immunohistochemistry-positive revealed point mutations that resulted in amino acid substitutions (Figure 7C). Overall, 16% of the clones contained mutations. A \rightarrow G or G \rightarrow A transitions were the most common

mutations observed and involved codons 23, 34, 72, 76, and 80 (Table 3). Other transition mutations included C \rightarrow T (codon 44), T \rightarrow C (codon 70), and A \rightarrow T (codon 56). Four of the clones from three different animals had mutations within the glycogen synthase kinase-3B (GSK-3B) phosphorylation domain at codon 34 and codon 44 (Figure 7, C and D). Of the four mutations within the phosphorylation domain, three resulted in substitution of an amino acid with a nonpolar side chain by an amino acid with a polar uncharged (Pro45Ser) or a polar charged (Gly34Glx) amino acid side chain. The fourth amino acid substitution within the phosphorylation domain retained the polar side chain but replaced a relatively small amino acid with a larger, space-occupying molecule (Gly34Ile). Seven other mutations resulted in amino acid substitutions in regions adjacent to the GSK-3B phosphorylation domain (Figure 7C). Of the mutations outside the phosphorylation domain, amino acid substitutions resulted in altered charge (Gln72Arg, Gln76Arg, Asx56Val), polarity

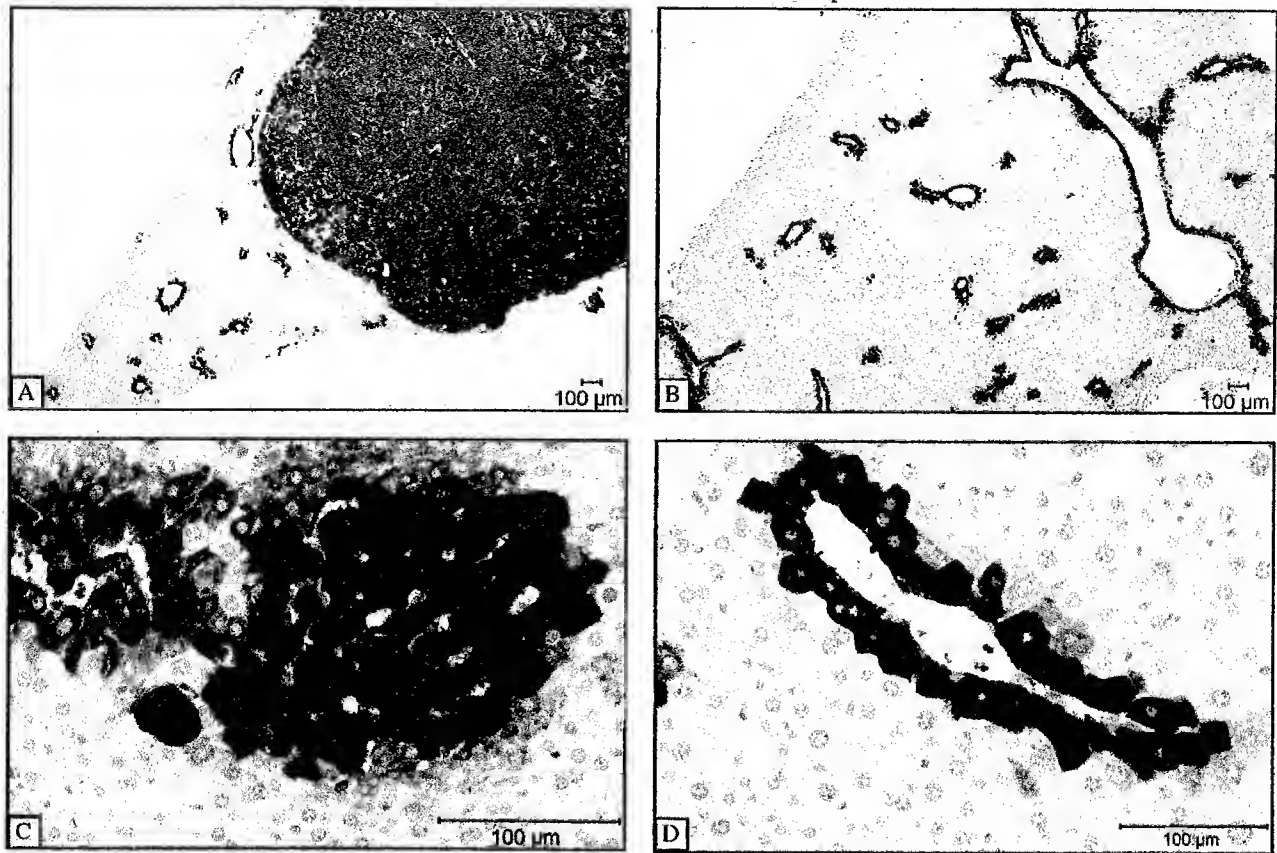


Figure 5. Glutamine synthetase immunoreactivity of dysplastic and neoplastic hepatocytes from FGF19 transgenics. **A:** Neoplastic cells are strongly glutamine synthetase-positive. **B:** Liver from a wild-type mouse showing normal perivenular glutamine synthetase immunostaining. **C:** Dysplastic hepatocytes are strongly glutamine synthetase-positive. **D:** Normal glutamine synthetase immunoreactivity of perivenular hepatocytes. Original magnifications: $\times 40$ (**A** and **B**), $\times 400$ (**C** and **D**).

(Ala80Ser, Ser23Gly), or molecular size (Phe70Leu). Mutations that affect GSK-3 β phosphorylation of β -catenin prevent ubiquitination and degradation,^{48,49} resulting in cytoplasmic accumulation and nuclear translocation of β -catenin, which accounts for the immunoreactivity observed in this study. Figure 7C shows the amino acid alignment of all mutant β -catenin clones compared to the wild-type sequence, depicting relative positions of amino acid substitutions and the GSK-3 β phosphorylation domain.

Discussion

We have demonstrated that overexpression of FGF19 at an ectopic site (skeletal muscle) leads to liver dysplasia and HCCs in transgenic mice. Preneoplastic changes including AFP expression and increased hepatocellular proliferation have been confirmed in a second founder line to rule out insertional activation of a dominant oncogene resulting from integration of the transgene. In this study, FGF19 transgenic mice develop HCCs by 10 to 12 months of age with a higher incidence in female transgenics (Table 1). In contrast, transgenic mice overexpressing TGF- α in the liver under control of the metallothionein-I promoter have a higher incidence of tumors in males.³⁶ There is also a male predominance of HCC in

humans in developed countries and worldwide.⁵¹ Therefore, understanding the reason for the higher incidence of HCC in female FGF19 transgenic mice may help elucidate the pathogenesis of HCC in other species.

In the normal liver, hepatocytes are mitotically quiescent, remaining in the G₀ phase of the cell cycle. However, chemical carcinogens including phenobarbital and peroxisome proliferators^{52,53} as well as overexpression of oncogenes in the liver²⁷ induce transient and/or sustained hepatocyte proliferation that precedes transformation. Although FGF19 is minimally mitogenic for fibroblasts *in vitro* relative to other FGF family members,¹⁴ hepatocellular proliferation was significantly elevated in nontransgenic mice injected with FGF19 protein (Figure 4E) and in 2- to 4-month-old FGF19 transgenic mice (Figure 4C). These are data consistent with previous observations that constitutive hepatocyte proliferation precedes liver tumor development, with or without liver damage, in other transgenic mouse models.²⁷

Mechanistic studies of hepatocarcinogens have identified specific morphological changes associated with unique molecular alterations.⁵⁴ For example, diethylnitrosamine induces pericentral foci of small dysplastic hepatocytes^{55,56} and acts by ethylating nucleophilic sites in DNA.⁵⁷ Diethylnitrosamine-induced HCCs are also glutamine synthetase-positive suggesting that the neoplas-

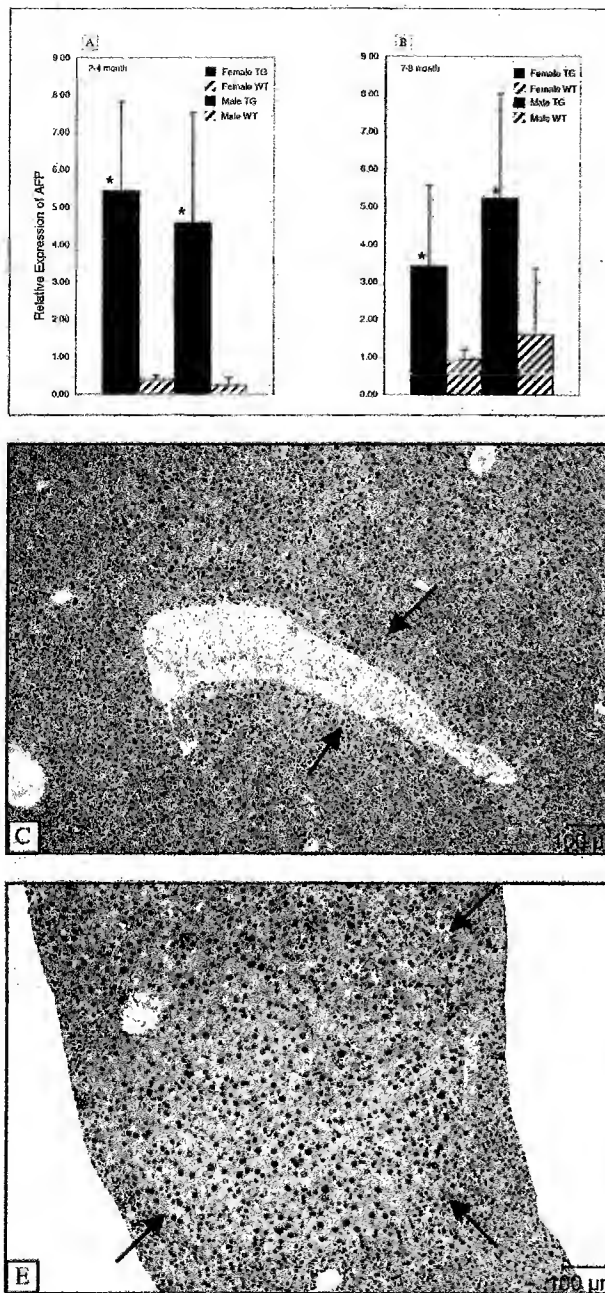


Figure 6. Expression of AFP by neoplastic and dysplastic hepatocytes. Increased expression of AFP mRNA in FGF19 transgenic liver compared to wild-type liver at 2 to 4 months of age (A) and 7 to 9 months of age (B). *, $P < 0.05$. Bright-field (C) and dark-field (D) illumination of *in situ* hybridization with AFP riboprobe showing expression of AFP by pericentral dysplastic hepatocytes (arrows). Bright-field (E) and dark-field (F) illumination of *in situ* hybridization with AFP riboprobe showing expression of AFP by neoplastic hepatocytes (arrows). Original magnifications, $\times 100$.

tic hepatocytes originated from the constitutive glutamine synthetase-positive population, pericentral hepatocytes.⁵⁸ Small dysplastic hepatocytes that oriented around the central veins were the predominant type of dysplasia that preceded neoplastic transformation in FGF19 transgenics (Figure 1C). Similarly, dysplastic and neoplastic foci in FGF19 transgenic mice were positive for glutamine synthetase (Figure 5). Selective involvement of pericentral hepatocytes may imply a potential pathogenesis of hepatocellular tumor formation such as DNA adduct formation that results from diethylnitrosamine. Alternatively, the pericentral hepatocytes that are highly metabolically active may be more susceptible to oxidative stress resulting in free radical formation, DNA damage, and subsequent tumor initiation. This concept

has precedence in the TGF- α /c-myc double-transgenic mouse model of HCC in which an environment of oxidative stress is present before tumor formation⁵⁹ and treatment with vitamin E, a potent free-radical scavenging antioxidant, prevents progression to HCC.⁶⁰

We have previously described an increased metabolic rate associated with decreased adiposity in FGF19 transgenic mice.⁴⁰ Whether HCC formation is an indirect effect of altered metabolism or a direct effect of FGF19 is unknown. Examples of mouse models in which altered metabolism leads to hepatocellular changes include liver dysplasia associated with loss of insulin signaling in hepatocytes of liver-specific insulin receptor knockout mice (LIRKO).⁶¹ The authors hypothesized that hepatocellular dysplasia was because of oxidative stress, however, peri-

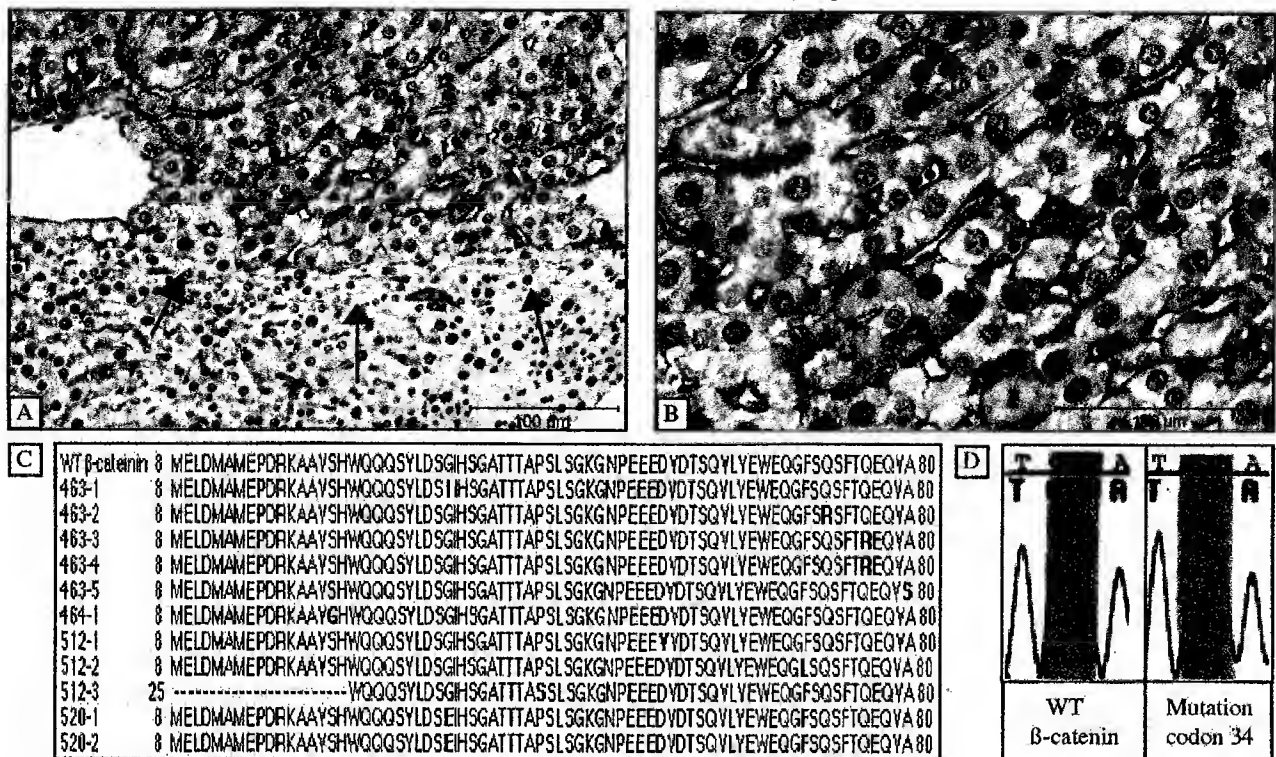


Figure 7. β -Catenin immunoreactivity of neoplastic hepatocytes from FGF19 transgenics. **A:** Strong nuclear staining of neoplastic cells compared with surrounding liver. **Arrows** mark the border of the tumor and adjacent normal liver. **B:** Neoplastic hepatocytes with nuclear immunoreactivity for β -catenin. **C:** Amino acid sequence alignment of the N-terminal region of β -catenin from wild-type (top) and mutant clones with amino acid substitutions (bold) in and adjacent to the GSK-3B phosphorylation domain (red). **D:** Sequencing data for DNA from normal liver and HCC with nucleotide substitutions at codon 34 (shaded). Original magnifications: $\times 200$ (A); $\times 400$ (B).

central hepatocytes were not preferentially affected in the LIRKO mice nor did the hepatocellular dysplasia described in their study progress to HCCs. Therefore, FGF19 may act directly as a tumor promoter instead of causing HCCs secondary to altered metabolism. Expression patterns of FGFR4, the receptor for FGF19,¹⁴ in murine liver (Figure 3)

Table 3. Nucleotide and Amino Acid Substitutions in β -Catenin Clones from FGF19 Transgenic HCCs that Have Nuclear Accumulation of β -Catenin Shown by Immunohistochemistry

Animal ID-clone number	Mutation	Codon	Amino acid change
463-1	(GG to AT)	34	Glycine to isoleucine*
463-2	(A to G)	72	Glutamine to arginine
463-3	(A to G)	76	Glutamine to arginine
463-4	(A to G)	76	Glutamine to arginine
463-5	(G to A)	80	Alanine to serine
464-1	(A to G)	23	Serine to glycine
512-1	(A to T)	56	Aspartic acid to valine
512-2	(T to C)	70	Phenylalanine to leucine
512-3	(C to T)	44	Proline to serine*
520-1	(GG to GA)	34	Glycine to glutamic acid*
520-2	(GG to GA)	34	Glycine to glutamic acid*
Total mutations	11/66 (16.6%)		

The animal identification followed by a hyphen and the clone number is indicated in the left column. The nucleotide substitution and affected codon are shown in the second and third columns, respectively. The amino acid change is shown on the right.

*The mutation was within the GSK-3B phosphorylation domain.

support the notion that FGF19 could directly activate hepatocytes.

Because expression levels of growth factors and oncogenes were not altered in the FGF19 transgenic mice (data not shown), we determined whether mutations in tumor suppressors contributed to the hepatocarcinogenesis. HCCs in transgenic mouse models overexpressing *c-myc* or *H-ras* and human HCCs have activating somatic mutations within the β -catenin gene.⁶²⁻⁶⁴ Mutations in β -catenin have also been identified in murine HCCs induced by diethylnitrosamine.⁶⁵ β -catenin is involved in cell adhesion and transmission of the proliferating signal of the Wntless/Wnt pathway.^{66,67} Mutations in the gene encoding β -catenin typically affect the phosphorylation site thereby inhibiting GSK-3B phosphorylation of β -catenin that prevents ubiquitination and degradation.^{49,50} Our results show that liver tumors from 44% (four of nine) of FGF19 transgenic mice had neoplastic cell nuclei that were immunoreactive with antibodies to β -catenin (Figure 7, A and B). Cloning and sequencing DNA from the FGF19 transgenic tumors clearly demonstrated point mutations leading to amino acid substitutions in and adjacent to the GSK-3B phosphorylation site of β -catenin (Figure 7C). Transition mutations resulting in amino acid substitutions in nine different codons were revealed (Table 3). A \rightarrow G or G \rightarrow A transitions were the most common mutations observed and involved codons 23 (Ser23Gly), 34 (Gly34Glu, Gly34Ile), 72 (Gln72Arg), 76

(Gln76Arg), and 80 (Ala80Ser). To our knowledge, only one of the mutations observed in the FGF19 transgenic HCCs, Gly34Glu, has been previously described in murine HCC.⁶⁴

β -Catenin mutation frequency is variable in different transgenic mouse models of liver cancer. Previous studies demonstrate β -catenin mutation frequencies of 5 to 12% in HCCs from *c-myc*, *c-myc/TGF- α* , and *c-myc/TGF- β* transgenic mice in which transgene expression is driven in the liver by the albumin promoter.⁶³ However, a higher β -catenin mutation frequency, 40 to 55%, occurs in HCCs from transgenic mice overexpressing H-ras or *c-myc* under control of the L-type pyruvate kinase (L-PK) promoter, and *c-myc* under control of the woodchuck hepatitis virus promoter.⁶⁴ β -catenin nuclear translocation in 44% of the FGF19 HCCs, with β -catenin mutations identified by sequencing DNA from these tumors, is in accordance with the β -catenin mutation frequency from the latter group of L-PK/H-ras, L-PK/*c-myc*, and woodchuck hepatitis virus/*c-myc* transgenic mouse models. The variability of β -catenin mutation frequency may be attributable to the different background strains as has been suggested,⁶³ or indicative of specific molecular mechanisms of hepatocellular transformation.

Taken together, these data suggest a previously unknown role for FGF19 in hepatocarcinogenesis. Hepatocellular proliferation in young transgenic mice and rFGF19 protein-injected mice implies FGF19 may directly affect hepatocytes. Expression of the receptor for FGF19, FGFR4, in murine liver supports this hypothesis. Moreover, our data implicate the Wntless/Wnt pathway in the molecular pathogenesis of HCCs in FGF19 transgenic mice. Further studies may define novel roles for FGF19 in carcinogenesis.

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